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Beta-2-adrenergic receptor polymorphisms in cystic fibrosis

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Abstract

Objectives—Cystic fibrosis (CF), an autosomal recessive disease affecting the lung, pancreas, gut, liver, and reproductive tract, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes a cyclic adenosine 3′, 5′ monophosphate-regulated chloride channel. The variability of disease progression among patients with CF suggests effects of genetic modifiers of disease. Beta-2 adrenergic receptors (β_2AR), which are abundant in airway epithelial cells, accelerate the formation of cyclic adenosine 3′, 5′ monophosphate, which can modulate *CFTR* activity and affect smooth muscle contractility. We tested the hypothesis that genetic variants of the *β_2AR* gene, which have been shown to influence receptor desensitization, are more frequent in patients than in controls.

Methods—We genotyped 130 adult CF patients and 1 : 1 age-matched, sex-matched, and ethnicity-matched normal volunteers for $Gly^{16}Arg$ and $Gln^{27}Glu$ *β_2AR*.

Results—We found that CF patients were more likely than controls to be Gly^{16} homozygotes (48 and 32%, respectively) ($P < 0.01$) and Glu^{27} homozygotes (29 and 10%, respectively) ($P < 0.01$).

Conclusions—Our results, showing a higher frequency of Gly^{16} and Glu^{27} *β_2AR* alleles in adult CF patients than in the control population, contrast with data from children with CF, who are reported to have lower frequency of Gly^{16} and similar frequency of $G1u^{27}$, and with data from young adults with CF, who showed no differences in frequencies of *β_2AR* variants. The $Gly^{16}Glu^{27}$ variant of *β_2AR* may have properties that lead to enhanced *β_2AR* function, resulting in the upregulation of *CFTR* activity and the improvement of CF disease.

Keywords

β_2-adrenergic receptor; bronchodilator response; cystic fibrosis; single nucleotide polymorphism

Introduction

Cystic fibrosis (CF), an autosomal recessive disease affecting the lung, pancreas, gut, liver, and reproductive tract, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride channel. The most common mutation in CFTR, ΔF508, occurs in approximately 70% of CF alleles in the Caucasian population [1], and results in a channel that has only about one-third of normal activity [2],

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is improperly processed and mislocalized in the cell [3], and is retrieved from the plasma membrane at a rate 10 times that of the wild-type protein [4]. Although CF is caused by mutations in CFTR, a growing body of data points to the importance of genes that may modify clinical features and course of the disease [5]. The CFTR protein, a cyclic adenosine 3′,5′ monophosphate (cAMP)-regulated channel, must be phosphorylated by protein kinase A (PKA) to release the regulatory or *R* domain of the protein, thus permitting the channel to open. The beta-2-adrenergic receptor (β_2-AR), a seven transmembrane-spanning G protein-coupled receptor, is activated by β-adrenergic agonists that stimulate the production of cAMP through the stimulation of adenylyl cyclase via the heterotrimeric guanine triphosphate-binding protein Gs. Beta-2-ARs have been found in a complex with CFTR and ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50), and β_2-ARs and CFTR have been colocalized at the apical membrane [6,7]. As some mutant CFTR channels retain the limited ability to conduct chloride and can be activated pharmacologically by compounds that activate or inhibit components of the PKA pathway, including β_2-AR, phosphodiesterases, adenylyl cyclase, phosphatases, and PKA [8,9], it is also possible that polymorphisms in the genes encoding these proteins may also stimulate CFTR activity through increases or decreases in activities of the components. An increase in β_2-AR activity would be predicted to result in an increase in CFTR activity via effects on cAMP. Such findings provide a theoretical rationale to consider genetic variants of the β_2-AR as gene modifiers in CF.

The β_2-AR gene is on chromosome 5q31–32 [10]. Several β_2-AR polymorphisms have been identified, with the two most widely studied located at nucleotide positions 46 and 79, relative to the start of translation [10]. An adenine at position 46 produces an arginine at codon 16 (Arg^{16}), whereas a guanine results in Gly^{16}. With cytosine at position 79, codon 27 encodes Gln^{27}, and a guanine encodes Glu^{27}. Arg^{16} is in tight linkage disequilibrium with Gln^{27}, such that $Arg^{16}Glu^{27}$ rarely occurs [10]. Neither polymorphism affects ligand binding or adenylyl cyclase-activating activity of the receptor [11], but agonist-promoted downregulation of the Gly^{16} receptor is enhanced relative to that of the Arg^{16} variant, whereas the Glu^{27} receptor resists downregulation *in vitro* [11]. In-vivo, however, greater desensitization of the Arg^{16} variant is seen in airways, vasculature, and cardiac tissue treated with a long-acting β-adrenergic receptor agonist [12–17], although this may be because the Gly^{16} variant is already highly desensitized because of endogenous catecholamines [15,18]. A greater response to isoproterenol in venodilatation is seen in people homozygous for Gly^{16}-Glu^{27} [14,15,17]. When response to long-term terbutaline was studied, those individuals homozygous for Arg^{16} and Gln^{27} and those homozygous for Gly^{16} and either Gln^{27} homozygotes or $Gln^{27}Glu$ heterozygotes showed greater agonist-induced desensitization as measured by effects on heart rate and contractility than those homozygous for Glu^{27} (with either $Gly^{16}Gly$ or $Arg^{16}Gly$) [16]. The Glu^{27} receptor has also been shown to be protective against asthma [19]. We questioned whether effects of β_2-AR polymorphisms might be different in adult patients with CF. Two previous studies of β_2-AR polymorphisms in CF populations have been published. Buscher *et al*. [20] assessed a young CF population (mean age approximately 13 years of age), with 60% ΔF508 homozygosity. In that cohort, the allelic frequency of Arg^{16} (0.61 for Arg16, 0.39 for Gly16) was higher than that in the controls, whereas allelic frequencies for codon 27 were similar to controls. In a subsequent study, Hart *et al*. [21] observed similar allelic frequencies for both codons in CF subjects (average age of 20 years with 63% ΔF508 homozygosity) versus controls. In the current study, we assessed the effects of the two β_2-AR polymorphisms on disease course in adult patients with CF.

Materials and methods

Study population

The research was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (NHLBI protocols 98-H-0062 and 01-H-0163), and informed consent was obtained from all participants. CF patients were 1 : 1 matched to normal research volunteers on the basis of ethnicity, sex, and age (± 5 years) (NHLBI protocol 96-H-100). CF patients were genotyped for 86 CFTR mutations (Genzyme Genetics, Westborough, Massachusetts, USA).

Genotyping

Genomic DNA was prepared from whole blood using the PureGene kit (Gentra Systems, Minneapolis, Minnesota, USA). A fragment containing the 16 and 27 polymorphisms was amplified with polymerase chain reaction using the primers 5′-ATGGGGCAACCCGGGAACGGCAGC-3′ and 5′-CTGCCAGGCCCATGACCAGATCAG-3′. Fragments were then sequenced using the primer 5′-CTTGGCAATGGCTGTGATGAC-3′ and the BigDye Terminator Sequencing kit (Applied Biosystems, Foster City, California, USA).

Pulmonary function testing

Pulmonary function testing was performed according to American Thoracic Society standards [14,15]. Forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were measured before and after administration of albuterol, either 2.5 mg via nebulizer or 180 μg via a metered-dose inhaler. A positive response to bronchodilators was defined as an increase of 12% and 200 ml in either FEV_1 or FVC [22,23].

Statistics

In this 1 : 1 age, sex, and ethnicity-matched case–control study, β_2-AR genotypes in CF patients and controls were compared, using Multinomial Logistic Regression in the statistical software SPSS. Linear Regression analyses (SPSS, Dallas, Texas 75248, USA) were performed to assess the relationships between the phenotypes and β_2-AR genotypes within the CF population. *T*-tests were performed with Microsoft Excel. Alpha < 0.05 was considered as statistical significance.

Results

Study population

One hundred and thirty CF patients were included in this study, 37% of whom were ΔF508 homozygous (Table 1). Slightly more than half (57%) were women, all but two were Caucasian (two African-Americans), and their mean age was 31 years. Controls were matched for age, sex, and ethnicity.

Analysis of beta-2-adrenergic receptors polymorphisms

We genotyped patients and matched controls for codon 16 and codon 27 polymorphisms of the β_2-AR gene. The genotype frequencies of the β_2-AR^{16} polymorphism were significantly different ($P < 0.01$) in the CF and normal volunteer populations, with a lower frequency of the Arg^{16} allele in the CF than in the control population (0.30 Arg^{16}, 0.70 Gly^{16} in the CF group, and 0.40 Arg^{16}, 0.60 Gly^{16} in the healthy controls) (Table 2). Genotype frequencies of the β_2-AR^{27} polymorphism were also significantly different ($P < 0.01$) in CF patients and controls, with a higher frequency of the Glu^{27} allele in the CF population than in the normal volunteers (0.48 Gln^{27}, 0.52 Glu^{27} in the CF group, and 0.68 Gln^{27}, 0.32 Glu^{27} in controls).

Pulmonary function testing and bronchodilator response

No significant difference was seen in FVC, FEV_1, or diffusing capacity for carbon monoxide (DL_{CO}) among the ΔF508 homozygous CF patients stratified by β_2-AR genotypes (Table 3). Many CF patients use inhaled bronchodilators, both for bronchodilation and mucociliary clearance [24,25]. Among individuals in the CF population, 36% responded to bronchodilators, whereas 46% of the ΔF508 homozygous patients responded. The ΔF508 homozygous patients who responded to bronchodilators had significantly lower percent-predicted FEV_1 than did the nonresponders (48 ± 4 and 64 ± 5, respectively; $P = 0.023$).

Frequencies of the β_2-AR polymorphisms were not different in bronchodilator responders and nonresponders (data not shown). The ΔF508 homozygous patients with the $Arg^{16}Arg$ genotype who did not respond to bronchodilators had significantly higher percent-predicted values for FVC and FEV_1 than did those with the $Arg^{16}Gly$ or $Gly^{16}Gly$ genotypes (FVC: $P < 0.01$; FEV_1: $P < 0.01$) (Table 4). Those nonresponders who were homozygous for Gln^{27} also had significantly higher percent-predicted FVC than did those with the Glu^{27} allele ($P < 0.01$). Among the ΔF508 homozygous patients who responded to bronchodilators, the percent-predicted FVC of patients with $Gln^{27}Glu$ was significantly higher than that of the homozygotes ($P < 0.01$) (Table 4), and the percent- predicted FEV_1 and DL_{CO} for the heterozygotes tended to be higher than those of either homozygote (FEV_1: $P = 0.054$; DL_{CO}: $P = 0.049$). It should be noted, however, that most of the subgroups of patients are small and thus, the possibility of sampling error exists.

Discussion

CF is caused by mutations in the CFTR, which encodes a cAMP-regulated chloride channel activated by phosphorylation by PKA. Events that regulate CFTR-mediated chloride permeability include phosphorylation of the regulatory or *R* domain of the protein and binding and, hydrolysis of ATP at the nucleotide-binding folds. Mutations in CFTR have been classified into five groups depending on the effect of the mutation, that is, CFTR synthesis, protein maturation, chloride channel regulation/ gating, chloride conductance, protein stability [26]. The most common mutation in CFTR, ΔF508, results in a channel with impaired activity that is mislocalized in the cell, although the amount of correctly localized ΔF508 CFTR varies in different cell types [26]. A mutant CFTR channel with defects in regulation/gating or chloride conductance that is transported to some degree to the plasma membrane may be stimulated pharmacologically by compounds that activate or inhibit components of the PKA pathway, including β_2-AR, phosphodiesterases, adenylyl cyclase, phosphatases, and PKA itself [8,9]. Polymorphisms in β_2-AR that stimulate β_2-AR function may also upregulate mutant CFTR activity, perhaps leading to a more favorable disease course.

β_2-AR and CFTR have been colocalized in the apical membrane of airway epithelial cells [6], where a macromolecular complex comprising CFTR, β_2-AR, and EBP50 has been identified [6,7]. CFTR and β_2-AR interact with EBP50 through PDZ-binding motifs in the C-termini of the proteins. Removal of the PDZ-binding motif from CFTR disrupted its interaction with EBP50 and β_2-AR and decreased chloride efflux through CFTR stimulated by β_2-AR activation [6]. CFTR phosphorylation by PKA inhibited the EBP50 binding, enabling its activation [6]. Thus, the dynamic complex of CFTR, EBP50, and β_2-AR provides for compartmentalized regulation of CFTR signaling.

Taouil *et al*. [7] found that incubation of airway epithelial cells with salmeterol, a long-acting β_2-AR agonist, increased levels of mature CFTR, which was not due to increased amounts of CFTR mRNA, cAMP, or PKA activity. Although β-agonists increased CFTR levels, those of EBP50 remained the same, and those of β_2-AR decreased. If β_2-AR and

CFTR compete for the same binding site on EBP50, polymorphisms that increase desensitization of β_2-AR and removal of β_2-AR from the membrane may also free EBP50-binding sites, thereby increasing the amount of CFTR on the apical membrane. As very little additional CFTR function is necessary to improve a patient's clinical phenotype [27], variation in β_2-AR polymorphisms may contribute to the clinical phenotype seen in our older cohort of CF patients.

Beta-2-AR polymorphisms have been studied *in vivo* in the airways, vasculature, and cardiac tissue [12–17,19,28–30]. Several studies have shown that Gly^{16} is associated with a more favorable response to the regular use of β_2-AR agonists, although *in vitro* data had shown greater downregulation of such receptors when incubated with agonists [11,30,31]. A meta-study on β_2-AR polymorphisms and asthma concluded that the Glu^{27} allele may be more resistant to downregulation than Gln^{27} [19]. Studies of β_2-AR polymorphisms in the vasculature demonstrated that greater maximal isoproterenol-stimulated dilation of a hand vein occurred before desensitization in those homozygous for Gly^{16} and Glu^{27} than in Arg^{16} and Gln^{27} or Gly^{16} and Gln^{27} homozygotes [15]. After chronic exposure to isoproterenol, participants who were homozygous for Arg^{16} showed almost complete desensitization, whereas those homozygous for Gly^{16} did not exhibit significant desensitization, irrespective of the allele at position 27 [14,15,17]. An explanation for these data is that Gly^{16}-containing receptors are significantly desensitized by endogenous catecholamines, as compared with Arg^{16} receptors, before chronic exposure to isoproterenol. Data on cardiac responses indicate that $Arg^{16}Gln^{27}$ participants show greater downregulation when treated with the agonist terbutaline than do participants with $Gly^{16}Gln^{27}$, who, in turn, show greater downregulation than do participants with $Gly^{16}Glu^{27}$ [16].

Such reports provide a useful background for the current findings related to β_2-AR variants in adult patients with CF. We found that the genotype frequencies of β_2-AR differed significantly in the CF and matched control population, with the frequencies of Gly^{16} and Glu^{27} significantly higher in the CF group. Genotype frequencies in the normal volunteer population are similar to data from the literature [20,21,31]. If CFTR and β_2-AR compete for binding sites on EBP50, the desensitization of the Gly^{16} variant by endogenous catecholamines may make extra sites available on EBP50 for CFTR. This would result in more CFTR available for activation at the plasma membrane. In the vasculature, receptors with the $Gly^{16}Glu^{27}$ variant produced a larger venodilative effect in response to acute β-agonist exposure than the $Gly^{16}Gln^{27}$ or $Arg^{16}Gln^{27}$ variants [15]. If this effect is similar in epithelial cells, then the $Gly^{16}Glu^{27}$ variant would also be able to produce more cAMP, thus leading to activation of CFTR through PKA phosphorylation.

Buscher *et al*. [20] found an increase in the frequency of the Arg^{16} allele compared with controls in a young population (average age 13 years of age), whereas Hart *et al*. [21] found no differences in allelic frequencies for either codon compared with controls, with an average age of 20 years. The CF population in our study was older than those in these studies with an average age 31 years and with a lower percentage of patients who were ΔF508 homozygotes. These results suggest an age-dependency of β_2-AR allelic frequencies in CF; however, as the studies draw on different groups of patients, this cannot be definitively stated. A selection bias may exist in our study on the basis of the ability of patients to travel to the research site, the inclusion of an adult population, and the recruitment from across the United States, especially because β_2-AR variants occur with different frequency among participants with different ethnicities [10,11,32]. The differences in genotype frequencies are not due to differences in ΔF508 homozygosity, as we found that the β_2-AR frequencies are similar when only the ΔF508 homozygous CF patients are considered.

In our CF population, 36% responded to bronchodilators, with a higher percentage (46%) among the ΔF508 homozygotes, but the genotype frequencies of the β_2-AR polymorphisms among bronchodilator responders and nonresponders were not different. Although FVC, FEV_1, and DL_{CO} did not differ among different β_2-AR genotypes of ΔF508 homozygous patients, among non-responders, those homozygous for Arg^{16} or Gln^{27} had significantly higher FVC values than did patients with a Gly^{16} or Glu^{27} allele. In addition, patients homozygous for Arg^{16} also had significantly higher FEV_1 than did $Arg^{16}Gly$ or $Gly^{16}Gly$ individuals. Hart *et al*. [21] found the $Gly^{16}Glu^{27}$ haplotype associated with a greater magnitude of response to bronchodilator. Both we and Hart *et al*. [21] found that bronchodilator responders had lower baseline FEV_1 values than nonresponders, but, in contrast to Hart *et al*. [21], we observed no association between magnitude of bronchodilator response and β_2-AR genotype.

In the ΔF508 homozygotes CF population that responded to bronchodilators, FVC was significantly higher for the $Gln^{27}Glu$ heterozygous group than the $Gln^{27}Gln$ or $Glu^{27}Glu$ homozygotes; FEV_1 and DL_{CO} were also higher, although of borderline significance. Buscher *et al*. [20] reported that ΔF508 homozygotes CF patients, who were homozygous for Arg^{16}, had significantly higher FVC, FEV_1, and mid-expiratory flow at 50% of vital capacity than those with at least one Gly^{16} allele. $Gln^{27}Gln$ individuals had higher FVC, FEV_1, and $MEF_{50\%VC}$ values than did $Gln^{27}Glu$ and $Glu^{27}Glu$ patients; the differences, however, were not significant. Buscher *et al*. [20] also reported that the presence of Gly^{16} was associated with a worse 5-year clinical course. In contrast, Hart *et al*. [21] did not find any association between β_2-AR genotype and rate of decline of FEV_1 over a 5-year period. Neither Buscher *et al*. [20] nor Hart *et al*. [21] examined the relationship between β_2-AR polymorphisms and pulmonary function by segregating the populations into bronchodilator responders and nonresponders. The major limitation of our study is the small sample sizes once the patient population is divided into subgroups on the basis of response to bronchodilators and genotypes. Although the differences have been found to be statistically significant, a larger study will be required to confirm the results.

In addition to their potential role as a modifier of CFTR function, β_2-adrenergic receptors, as a consequence of their ability to raise cellular cAMP levels, may also influence activity and/or number of amiloride-sensitive epithelial sodium channels (ENaC), which can increase the Na+ transport and fluid clearance in the airway [33,34]. Thus, if ENaC were to contribute to the regulation of airway function in CF, genetic variants of β_2-AR would be predicted to modulate such regulation and would likely contribute to alveolar fluid accumulation and clearance, especially in injured lungs [35,36]. Although poor Na+ conductance has been suggested to contribute to reduced salt absorption in CF [37], recent studies of airways indicate that CF glands do not demonstrate excessive, ENaC-mediated fluid absorption [38]. Thus, the actual role of β_2-AR variants in regulation of ENaC remains to be determined [13].

The notion that modifying genes influence the clinical manifestations of CF is an important and timely issue [5]. As its role as an activator of cAMP formation and ability of cAMP/PKA to regulate CFTR function, the β_2-AR is potentially one such disease modifier. Beta-2-AR polymorphisms may influence the CF disease process by regulating responses of smooth muscle cells to bronchodilators or permitting direct CFTR activation in airway epithelial cells. β_2-AR polymorphisms have been studied predominately in the context of smooth muscle cells, that is, their effects on asthma or the vasculature, whereas CFTR has been studied mainly in epithelial cells, where the effects of the β_2-AR polymorphisms have not been studied rigorously. The latter may be a useful, future line of investigation.

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Table 1

Characteristics of patients with cystic fibrosis and matched normal volunteers

	CF	Controls
Number	130	130
Gender (women : men)	74:56	74:56
Age (years, mean ± SEM, range)	30.8 ± 0.8 (18–58)	30.8 ± 1.0 (17–64)
CFTR ΔF508 homozygous	36.9%	NA
CFTR ΔF508 heterozygous	50.0%	NA
Others	13.1%	NA
FVC (% predicted, mean ± SEM, range) [*n*]	76.6 ± 1.8 (26–119) [126]	NM
FEV_1 (% predicted, mean ± SEM, range) [*n*]	63.1 ± 2.3 (19–124) [129]	NM

NA: not applicable; NM: not measured; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second.

Table 2

Genotype frequencies of β_2-AR polymorphisms in CF matched to normal volunteers (NV)

		CF (%)	NV (%)
β2-AR16 *a*	Arg/Arg	10 (7.7)	17 (13.1)
	Arg/Gly	58 (44.6)	71 (54.6)
	Gly/Gly	62 (47.7)	42 (32.3)
β2-AR27 *b*	Gln/Gln	32 (24.6)	59 (45.4)
	Gln/Glu	61 (46.9)	58 (44.6)
	Glu/Glu	37 (28.5)	13 (10.0)

CF, cystic fibrosis; NV; normal volunteers.

a Frequencies of β_2-AR16 genotypes are significantly different in the CF population versus normal volunteers, $P < 0.01$.

b Frequencies of β_2-AR27 genotypes are significantly different in the CF population versus normal volunteers, $P < 0.01$.

Table 3

Percent predicted values of FVC, FEV_1, and DL_{CO} of CF ΔF508 homozygous patients

	FVC (mean ± SEM)	FEV_1 (mean ± SEM)	DL_{CO} (mean ± SEM)	*n*
β_2-AR[16]				
Arg/Arg	79 ± 11	74 ± 17	96 ± 12	4
Arg/Gly	70 ± 5	55 ± 5	96 ± 6	18
Gly/Gly	74 ± 4	56 ± 4	99 ± 4	26
β_2-AR[27]				
Gln/Gln	72 ± 7	61 ± 10	95 ± 11	10
Gln/Glu	75 ± 3	56 ± 4	99 ± 4	23
Glu/Glu	71 ± 5	56 ± 5	97 ± 6	15

FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; $DL_{CO,}$ diffusing capacity for carbon monoxide.

Table 4

Percent predicted values of FVC, FEV_1, and DL_{CO} of CF ΔF508 homozygous patient bronchodilator nonresponders and responders

	FVC (mean ± SEM)	FEV_1 (mean ± SEM)	DL_{CO} (mean ± SEM)	*n*
Nonresponders				
β_2-AR[16]				
Arg/Arg	93 ± 2*	95 ± 4**	109 ± 7	2
Arg/Gly	68 ± 8	58 ± 8	97 ± 10	6
Gly/Gly	78 ± 4	61 ± 6	104 ± 6	12
β_2-AR[27]				
Gln/Gln	91 ± 3@	80 ± 14	119 ± 11	3
Gln/Glu	74 ± 5	59 ± 6	99 ± 6	12
Glu/Glu	74 ± 7	64 ± 9	101 ± 12	5
Responders				
β_2-AR[16]				
Arg/Arg	46	24	63	1
Arg/Gly	73 ± 8	55 ± 8	98 ± 8	7
Gly/Gly	63 ± 5	45 ± 5	89 ± 6	9
β_2-AR[27]				
Gln/Gln	51 ± 6	34 ± 9	73 ± 14	3
Gln/Glu	77 ± 6#	56 ± 7##	100 ± 7###	9
Glu/Glu	57 ± 5	42 ± 3	87 ± 5	5

FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; DL_{CO}, diffusing capacity for carbon monoxide.

*$P < 0.01$ Arg/Arg versus Arg/Gly and Gly/Gly.

**$P < 0.01$ Arg/Arg versus Arg/Gly and Gly/Gly.

@$P < 0.01$ Gln/Gln versus Gln/Glu and Glu/Glu.

#$P < 0.01$ Gln/Glu versus Gln/Gln and Glu/Glu.

##$P = 0.054$ Gln/Glu versus Gln/Gln and Glu/Glu.

###$P = 0.049$ Gln/Glu versus Gln/Gln and Glu/Glu.